Available online at www.sciencedirect.com
ScienceDirect
Molecular and Cellular Probes 20 (2006) 366–370
Molecular and Cellular Probes
www.elsevier.com/locate/ymcpr

Diagnosis of "maladie des feuilles cassantes" or brittle leaf disease of date palms by detection of associated chloroplast encoded double stranded RNAs

A. Namsi[a], J. Marqués[b,d], Z. Fadda[b,d], M.L. Takrouni[a], O. Ben Mahamoud[a], A. Zouba[a], J.A. Daròs[b], R. Flores[b], J.M. Bové[c], N. Duran-Vila[d,*]

[a]*Institut National de la Recherche Agronomique de Tunisie, Centre de Recherches Phoenicicoles, 2260 Degache, Tunisie*
[b]*Instituto de Biología Molecular y Celular de Plantas (CSIC-Universidad Politécnica de Valencia), 46022 Valencia, Spain*
[c]*UMR 1090, Institut National de la Recherche Agronomique and University of Bordeaux 2, Villenave d'Ornon, France*
[d]*Departamento de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA). Apartado Oficial, 46113 Moncada (Valencia), Spain*

Received 26 April 2006; accepted 5 May 2006
Available online 19 May 2006

Abstract

The "Maladie des feuilles cassantes" (MFC) or "Brittle leaf disease" of date palms is associated with the accumulation of two populations of small, chloroplast-encoded RNAs. A plasmid vector containing a cDNA with partial sequences of both of these RNA populations was used to synthesize a DIG-labeled bifunctional probe by PCR. The probe has been tested to detect, by molecular hybridization, MFC-associated RNAs from dsRNA-enriched palm leaflet preparations. Leaflet samples from MFC-affected date palm trees consistently gave a positive hybridization signal regardless of the date palm cultivar, severity of symptoms, or geographical location, whereas samples from date palm trees affected by other biotic and abiotic stresses tested negative. The assay is specific for MFC and can be used for early diagnostic purposes.

Keywords: *Phoenix dactylifera*; Date palm trees; Brittle leaf disease; Manganese deficiency; Double stranded RNA; Diagnosis

1. Introduction

The "Maladie des feuilles cassantes" (MFC) or "Brittle leaf disease" is a disorder affecting date palms in Tunisia [1]. The leaflets of young palms of MFC-affected trees become brittle and break when flexed and squeezed. These symptoms eventually extend to the upper fronds, until the whole tree becomes affected, stops growing, becomes stunted, and eventually dies. The disease has been identified on most Tunisian cultivars, and in the last decade has taken alarming proportions, especially in the Nafta oasis [2] with 35,100 diseased trees. Recently, the disease has been reported to also occur in Algeria [3] and Lybia (Edongali et al., unpublished).

Mineral analysis has shown that adult leaflets from MFC-affected palm trees are severely deficient in Mn, suggesting that MFC is a nutritional disorder. However, other observations suggest horizontal transmission from an initially affected tree to neighboring trees, indicating a clustering and a non-random spread. Such a pattern is rather characteristic of biotic diseases, although no pathogen has been found yet [1].

Preliminary studies on the etiology of MFC, aiming at the identification of putative virus or virus-like agents, revealed the presence of two populations of host-encoded, single-stranded and double-stranded RNAs (MFC–RNAs) in symptomatic adult leaflets from affected plants (Marqués, Fadda, Flores, Duran-Vila and Daròs; unpublished

*Corresponding author. Tel.: +34 963424066; fax: +34 963424001.
E-mail address: nduran@ivia.es (N. Duran-Vila).

0890-8508/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2006.05.001

results). MFC–RNA sequences correspond to two different regions of date palm chloroplast genome, *rrn5s*/*trnR* and *ATPE*, coding for 5S rRNA, tRNA–Arg and ε subunit of ATP synthase, respectively. Despite it is not currently known the relation between these RNAs and the disease etiology, they are molecular markers of MFC and their detection is useful for diagnosis purposes. Availability of methods for MFC diagnosis are critical to investigate the spreading pattern of the disease and to develop control strategies. The objective of the present study was to establish a protocol for routine detection of MFC–RNAs in date palms by molecular hybridization and to evaluate its suitability for MFC diagnosis.

2. Materials and methods

2.1. Plant materials

Initial assays were performed with leaflet samples of the cultivar 'Deglet Nour'. Diseased samples were collected from severely MFC-affected trees in Nafta (Jouali oasis) and healthy controls from a modern plantation of the *Societé civile de mise en valeur agricole*, and from the Germplasm Bank in Degache, Tunisia, where diseased plants have never been observed. In some instances, samples from date palm trees growing in the oasis of Elche, Spain, were also included as negative controls. Further assays were done with leaf samples of different cultivars ('Deglet Nour', 'Kenta', 'Alig', 'Kentichi', 'Tezerzit' and 'Besser') collected in the affected oases of Nafta (Corbeille, Jouali and Fatnassa), Tozeur (Helba), Hamma (Hammam), Kebili (Fatnasa) and Tamerza (Brick) (Table 1).

Leaflet samples were also collected from six areas presenting different degrees of MFC incidence: Hamma (Hamman), severely affected with a high rate of diseased trees; Tozeur (Helba) and Nafta (Remada) both moderately affected; Kebili (Fatnassa), weakly affected; Tamerza (Brick), very slightly affected; and Nafta (*Société civile de mise en valeur agricole*, Essif Lakhdar), unaffected. Samples were collected from trees showing severe, moderate and weak symptoms, as well as from apparently healthy trees growing in the vicinity of others affected.

Samples were also collected from trees affected by other biotic and abiotic stresses in Tunisia and Morocco.

2.2. RNA extraction methods

Total RNA was extracted following the SDS/potassium acetate method [4,5]. Briefly, foliar tissue samples (500 mg) were homogenized inside sealed plastic bags in extraction buffer (0.1 M Tris–HCl, pH 8.0; 50 mM EDTA; 0.5 M NaCl; 10 mM 2-mercaptoethanol). The homogenates were incubated first in 1% SDS (65 °C for 20 min) and in 1 M potassium acetate (on ice for 20 min). RNAs in the soluble fraction were concentrated by ethanol precipitation and resuspended in 50 μl of water. RNA preparations were further purified by non-ionic cellulose chromatography for dsRNA enrichment [6,7]. Specifically, the preparations were resuspended in STE (100 mM NaCl; 50 mM Tris–HCl, pH 7.2; 1 mM EDTA) containing 17% ethanol and 50 μg of CF-11 cellulose (Whatman). The cellulose was washed twice with STE-17% ethanol and the dsRNAs bound to the cellulose were eluted with STE. The dsRNAs were concentrated by ethanol precipitation and resuspended in 50 μl of water.

2.3. Synthesis of a digoxigenin (DIG)-labeled probe

A bifunctional DNA probe was synthesized by PCR amplification in a final volume of 50 μl containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl and 1 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Roche), 120 μM each of the four dNTPs (including DIG-labeled dUTP) and 0.5 μM each of primers P1 (5′-CCAAAATGCAAAGGTCGTCTTAT-3′) and P2 (5′-GCTCGTCTGAGAGCTAGATTCG-3′). Plasmid pG MFCZ (2 ng) that contains in tandem two chloroplast date palm DNA inserts was used as a template. Date palm chloroplast DNA inserts correspond to the regions homologous to positions 98,642–98,886 or 116,233–116,476 (duplicated) and 51431–51592 of rice chloroplast genome (database accession number X15901). PCR parameters consisted of a denaturation step at 94 °C for 5 min, followed by 35 cycles of amplification (94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min), to finish with an extension step at 72 °C for 5 min. The synthesis of a DNA product of

Table 1
Detection of MFC-RNAs by molecular hybridization in palms showing different degrees of MFC symptoms

Cultivar	Positive samples/number of tested samples				Location
	Symptoms				
	Severe	Moderate	Weak	Symptomless[a]	
Alig	5/5	3/3	2/3	0/6	Kebili
Kenta	5/5	5/5	1/1	2/3	Hamma
Deglet Nour	12/12	13/13	9/9	13/39	Nafta, Hamma, Tamerza, Kebili, Tozeur
Total	22/22	21/21	12/13	15/49	
(%)	100	100	92.3	30.6	

[a]When positive hybridization was obtained with symptomless samples collected from apparently healthy trees growing in the vicinity of affected trees, they were weak (Hamma and Tamerza) or very weak (Tozeur).